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CYTOTOXIC EFFECT OF PARA HYDROXY META METHOXY CHALCONE (*p*H*m*MC) ON MCF-7 BREAST CANCER CELLS BY INDUCING CELL ARREST AND APOPTOSIS

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Abstract

Chalcones (1,3-diphenylpropen-1-ones), a biosynthetic product of the shikimate pathway, belonging to flavanoid family are precursors of open chain flavonoids and isoflavonoids, which are abundant in edible plants. These compounds have been studied as therapeutics, especially as antitumor drugs. A chalcone derivate, para hydroxy metha methoxy chalcone (*p*H*m*MC) or 3 - (4'-hydroxy-3'-methoxyphenyl)-1-phenyl-2-propene-1-on was synthesized by reaction between vanillin and acetofenon through cross-aldol condensation reaction under acidic conditions. The aim of this study is to investigate the cytotoxic effect of *p*H*m*MC on MCF7 breast cancer cells and its mechanism through investigation on apoptosis and cell cycle arrest. The cytotoxic effect on MCF-7 was analyzed by MTT [3-4, 5 dimetiltiazol-2-yl)-2,5difeniltetrazoilium bromide] assay. Flowcytometry method was used to determine the influence of *p*H*m*MC in the regulation of cell cycle arrest and apoptosis.The result showed that the *p*H*m*MC inhibited MCF-7 cell growth with IC_{50} 40 μM. The cytotoxic activity is influenced by the ability of *p*H*m*MC to induce apoptosis and G_2/M arrest.

Key words: *p*H*m*MC, cytotoxic effect, apoptosis, cell cycle arrest, and MCF-7 breast cancer cells

INTRODUCTION

Cancer is a serious public health issue in the world. One of the most five deadly cancer diseases leading patient death is breast cancer (WHO, 2006). In Indonesia, breast cancer is the second most frequently cancer in women after cervical cancer (Tjindarbumi and Mangunkusumo, 2002). There are several methods of cancer treatment such as surgery, radiation, immunotherapy, and chemotherapy (King, 2000). However, the high mortality rate indicates the treatments have not overcome the cancer. These problems pushed to find compounds for the cancer treatment either comes from natural or synthetic.

Chalcones, a group of aromatic enones, forms the central core a variaty of important

biological compounds obtained from plants. These phenoli compounds all bear a 1,3-diphenyl-2-en-1-one framework (Bohm, 1998). The synthetic chalcones can be prepared by an aldol condensation between a benzaldehyde and acetophenone in the presence of strong bases such as NaOH, KOH, $Ba(OH)_2$, hydrotalcites, LiHMDS, calcined NaNO3/natural phosphate. There are also some reports of acid-catalized aldol condensations, e.g. $AlCl_3$, BF_3, dry HCl, $ZrH_2/NiCl_2$ and $RuCl_3$ (for cyclic and acyclic ketones) (Rahman, 2011). Chalcones and its derivates have attracted increasing attention due to numeorous pharmacological applications. They have displayed a broad spectrum of pharmacological activities, one of them as anticancer (Achanta, et al., 2006; Romagnoli et al., 2008; Echeverria et al., 2009; Szliszka et a.l, 2010; Ilango et al., 2010; Arianingrum et al., 2011; Arianingrum et al., 2012).

A chalcone derivat compound, *p*H*m*MC or 3 - (4'-hydroxy-3'-methoxyphenyl)-1-phenyl-2-propene-1-on (Figure 1) was synthesized by reaction between vanillin and acetofenon through cross-aldol condensation reaction under acidic conditions (Arty, 2010). Previous study has reported that this compound induces apoptosis and inhibit phase of the cell cycle through G_2/M in breast cancer T47D cell line (Arianingrum et al., 2012). In the study, we investigated the effect of *p*H*m*MC on cytotoxicity, apoptosis induction and cell cycle of breast cancer MCF-7 cell lines. This study is useful for further development of *p*H*m*MC as medicine in the tratment of breast cancer.

RESEARCH METHOD

Material

The compound, *p*H*m*MK, was obtained from Prof Indyah Sulistyo Arty, Faculty of Mathematic and Natural Science, Universitas Negeri Yogyakarta (UNY). This compound was synthesized by reacting of vanillin and acetofenon through cross-aldol condensation reaction in acidic condition. The compound was diluted in dimethylsulfoxide (DMSO) at the desired concentration. The final DMSO concentration was made with concentration of less than 0.2%.

Cells Culture

MCF7 breast cancer cells were obtained from the collection of Laboratory of Parasitology, Faculty of Medicine, Universitas Gadjah Mada (UGM). Cells were grown in medium culture DMEM (Dulbecco's modified Eagle's Medium) from Gibco contains FBS (Fetal Bovine Serum, Gibco) 10% and penicillin-streptomycin 1% (Gibco) at 37ºC in humidified atmosphere of 5% CO_2/95% air. Tripsin-EDTA 0.025% (Gibco) was used to detached cells on the flask.

Cytotoxic Assay

Cytotoxic assay were performed using [3-4, 5 dimetiltiazol-2-yl)-2,5difeniltetrazoilium bromide] (MTT) assay. MCF7 cells were seeded at a density of 5×10^3 cells/well and allowed to attach for 24 hours. One day after initial seeding, cell were treated with either various concentration of *p*H*m*MC. After 24 hours of incubation, culture medium was removed and cells were washed with Phospate Buffered Saline (PBS) from Sigma. Then, cells were added with 100 mL MTT (Sigma Chemical, St. Louis, MO, USA) 5 mg/ml diluted with culture medium in each well for 4 hours. Stopper reagent , 10% Sodium dodecyl sulphate or SDS (Sigma) in 0.01 mol HCl (Merck) was added after formazan formation prior to MTT reduction and incubated for 12 hours (overnight) at room temperature and protected from light. The absorbance of each well was measured using ELISA reader (Bio-Rad) at λ 595 nm. The absorbance was converted to percentage of viable cells (Mosmann, 1983). IC_{50} concentration were calculated by the concentration that causes 50% inhibition of cell growth (Doyle and Griffith, 2000). Calculation of IC_{50} values were done using the linear regression of log concentration versus cells viability.

Apoptosis and Cell Cycle Analysis

MCF7 cells were seeded at 1 x 10^6 cells/well on six wells tissue culture plate. After 24 hours incubation cells were treated with *p*H*m*MK. After 24 hours of these treatments, cells were trypsinized, spin at 2000 rpm for 3 minute, and washed twice with cold PBS. Cells were resuspended in 500 µL of Annexin V buffer (Roche) and then treated with Annexin V and propidium iodide (PI) for 10 minute at room temperature and protected from light. The treated cells were subjected to FACScan flowcytometer. Bivariant analysis of FITC-flourosence (FL-1) and PI-flouresence (FL-3) gave different cell population where FITC (-) and PI (-) were designed as viable cells; FITC (+) and PI (-) phenotype as early apoptotic cells; FITC (-) and PI (+) as necrotic cells; and FITC (+) and PI (+) as late apoptotic cells. Cell cycle analysis was determined using cycletest™ Plus DNA Reagent Kit (BD Biosciences). Based on DNA contents, percentage of cells in each stage of cell cycle (G1, S and G_2/M phases) were calculated measured using Cellquest programe.

RESULT AND DISCUSSION

Cytotoxic effect of *p*H*m*MC on MCF-7 cells

MTT assay was done to measure cell viability of MCF-7 due to *p*H*m*MC treatment. This compound showed growth inhibitory effect in dose dependent manner (Figure 2 A-E). Based on morphological appearance and cells viability, *p*H*m*MC with concentration of 10, 25,50, 75, 100 and 125 µM respectively showed a linear correlation between concentration with toxicity effects. The IC_{50} value of *p*H*m*MC was 40 µM obtained from the linear regression calculation of cell viability versus log concentration with $P<0.05$. These effect supposed to be related to apoptotic induction and cell cycle modulation.

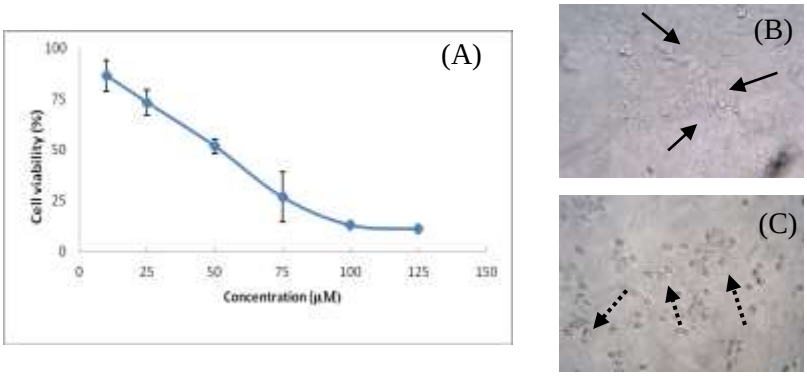

Figure 2. Cytotoxic effect of *p*H*m*MC on MCF-7 cell using MTT assay. Test were carrried out by incubating 10^4 cells in 96 well plates for 24 hours in DMEM medium without or with *p*H*m*MC treatments (0-125µM). Profiles of cell viability expressed mean ± SD of three experiments (A). Obvious morphological changes and cell populations in the treatment of *p*H*m*MC concentrations of 75 µM (C) compared with controls (B). Black arrow indicates a normal living cell, whereas dashed arrows indicate the cell morphology changes. Cell morphology observations conducted using an inverted microscope with 100x magnification.

Effect of *p*H*m*MC on apoptosis induction

Evaluation of the impact of *p*H*m*MC treatment on apoptosis induction of MCF-7 cells was detected using flowcytometric. This methods is detected translocation of phosphatidyl

serine residues which are normaly located in the internal phospholipid layer to external layer in apoptotic cells. The result showed that *p*H*m*MC induced apoptosis rates of early apoptotic cells and late apoptotic cells on MCF-7 cells (Figure 3). The percentage of apoptotic cells increased after treatment with *p*H*m*MC. Increased concentrations of *p*H*m*MC lead to more cells undergoing apoptosis.

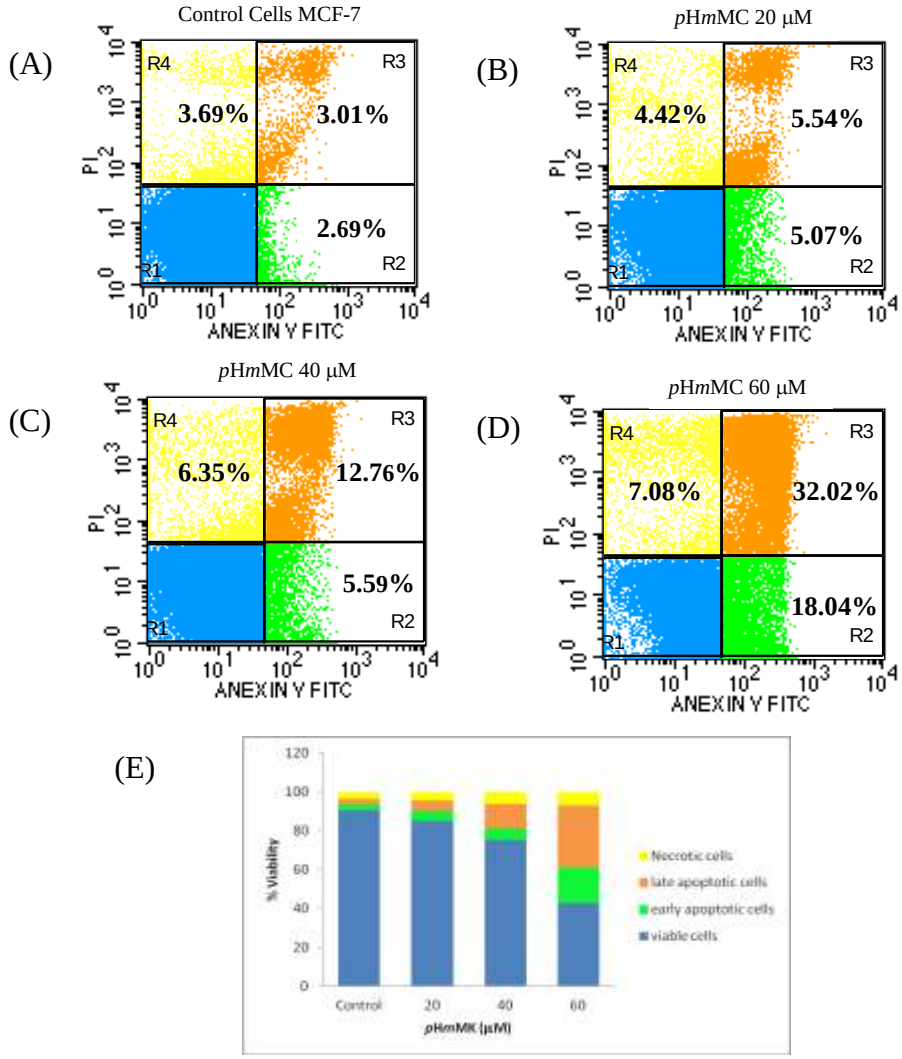

Figure 3. Effect of *p*H*m*MC on apoptosis induction in MCF-7 cells. The cells were seeded at $5x10^5$ cells/well on six wells tissue culture plate, then treated with *p*H*m*MC concentration of 0, ½ IC_{50}, IC_{50}, and 1½ IC_{50}. After 24 hours incubation, cell were harvested as described in methods, added AnnexinV and PI reagent, then subjected to FACS flowcytometer. Flowcitometric profile of cells treatment with 0 or control (A), 20 (B), 40 (C), and 60 (D) µM respectively. There are 4 quadrans : lower left (R1) marked in blue indicates viable cells, lower right (R2) marked in green indicates early apoptotic cells, upper right (R3) marked in orange indicates late apoptotic cells and upper left (R4) marked in yellow indicates necrotic cells. Graph (E) showed that *p*H*m*MC induced apoptosis.

Effect of *p*H*m*MC on cycle cells

To confirm that treatment of *p*H*m*MC increased cell death by modulating cell cycle, we evaluated cell cycle profile using flowcytometry method. Treatment with *p*H*m*MC induced cells

accumulation in G_2/M (Table 1).

Table 1. Percentage of MCF-7 cell at GO-G1, S and G2/M respectively after treatment with *p*H*m*MC at concentration 20 μM.

Sample	Persentage MCF-7 cells at phase: (%)		
	Go-G1	S	G_2/M
Control Cell	61.79	19.48	17.13
***p*H*m*MC**	64.89	13.79	19.26

Based on our research, *p*H*m*MC showed potent cytotoxic effect with IC_{50} value of 40 μM. Prayong et al (2008) mentioned that compound with IC_{50} less than 100 μM is potentially developed as anticancer agent. This result showed the potential of *p*H*m*MC to be delevoped as anti cancer.

As we know, that cancer cells are able to avoid apoptosis mechanism and cells occurs abnormal regulation of the cell cycle. The cytotoxic effect is suggested to be related to apoptotic induction and cell cycle modulation. In apoptosis study, in line with cytotoxic study *p*H*m*MC increased the incidence of cell undergoing apoptosis. Apoptosis is programmed cell death characterized by changes on morphology, membrane blebbing and chromatine (Rudin and Thomson, 1997). In this study, the morphological change from normal to dead of the cells were more extensive after treatment with *p*H*m*MC. In the flowcytometry analysis showed that treatment with *p*H*m*MC at 40 μM increased cell death from 5.7% to 18.35%, and increased to 50.06% after treatment with 60 μM *p*H*m*MC. These results indicate that this compound is able to induce apoptosis.

The cell cycle analysis was performed by using flowcytometer to observe the distribution of cells in each phase of the cell cycle. This method based on DNA content in cells. After the cells were incubated with propidium iodide, a red fluorescent DNA will be captured by the detector flowcytometer. Cell Cycle consists of proliferative phase, in the resting state (no cells divides, Go), and not permanently divide. Cells that are dividing divided into 4 (four) major phases: the gap phase 1 (G1), synthesis phase (S), gap phase 2 (G2), and mitosis phase (M) (Foster et al., 2001 and Vermeulen et al., 2003). In our research showed that treatment with *p*H*m*MC influence the cell cycle checkpoint control of MCF-7 by inducing G_2/M cell cycle arrest. The results of this analysis showed the compound influence on cell cycle checkpoint control.

Based on literature study, the basic structure of chalcone (1,3-diphenyl-2-propenone) has proven to have a chemopreventive effect in human breast cancer cell lines: MCF-7 and MDA-MB-231 (Hsu et al., 2006) and human bladder cancer cell lines: T24 and HT-1376 (Shen et al., 2007). The research showed that chalcone inhibits the proliferation of T24 and HT-1376 cells by inducing apoptosis and blocking cell cycle progression in the G2/M phase. Chalcone significantly increases the expression of p21 and p27 proteins, and decreases the levels of cyclin B1, cyclin A and Cdc2, thereby contributing to cell cycle arrest. In addition, chalcone increased the expression of Bax and Bak, but decreased the levels of Bcl-2 and Bcl-XL and subsequently triggered mitochondrial apoptotic pathway (release of cytochrome c and activation of caspase-9 and caspase-3). Chalcone also has NF-kB inhibitor activity at concentration of 50 μM (Shen et al, 2007). Nuclear factor kappa B (NF-κB) is transcription factors that plays a major role in development and progression of cancer because it regulates more than 400 genes involved in inflammation, cell survival, cell proliferation, invasion, angiogenesis, apoptosis, cell cycle and

metastasis (Yadav et al., 2011 and Pahl, 1999).

Our compound, *p*H*m*MC, is chalcone derivat that have -3' metoxy and -4' hydroxyl phenyl. Although there are modifications to the structure, this compound still has a cytotoxic activity. But in this study, we did not compare the activity with the basic compound of chalcone. However the changes of the structure will affect its activity. Our present study showed that *p*H*m*MC increased apoptosis induction via G_2/M arrest in MCF-7 cells. We proposed that NF-κB survival system may play important roles in the antiproliferative activity of *p*H*m*MC in MCF-7cells, but this mechanism need to be explored further. However, the molecular mechanism of apoptotic induction and cell cycle need to be explored with more details.

CONCLUSION AND SUGGESTION

Based on the result, we concluded that *p*H*m*MC has activity cytotoxic throught apoptosis induction and cell cycle arrest. This compound is potential to be develop as chemotherapeutic agent in breast cancer therapy

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